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1) Structure Fold Des 2000 Jun 15;8(6):565-6
Re-engineering ketoacyl synthase specificity.
Val D, Banu G, Seshadri K, Lindqvist Y, Dehesh K.

2) J Biol Chem 1980 Dec 25;255(24):11949-56
Structural, enzymatic, and genetic studies of beta-ketoacyl-acyl carrier protein synthases I and II of Escherichia coli.
Garwin JL, Klages AL, Cronan JE Jr.

3) Chem Biol 1997 Oct;4(10):757-66
Molecular recognition of diketide substrates by a beta-ketoacyl-acyl carrier protein synthase domain within a bimodular polyketide synthase.
Chuck JA, McPherson M, Huang H, Jacobsen JR, Khosla C, Cane DE.

4) Curr Opin Biotechnol 1997 Aug;8(4):429-34
Engineering novel proteins by transfer of active sites to natural scaffolds.
Vita C.

Thank you,
David Steadman

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 24, Issue of December 25, pp. 11949–11956, 1980
Printed in U.S.A.

Structural, Enzymatic, and Genetic Studies of β-Ketoacyl-Acyl Carrier Protein Synthases I and II of *Escherichia coli* *

(Received for publication, July 22, 1980)

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β-Ketoacyl-acyl carrier protein synthases I and II of *Escherichia coli* were purified and characterized. Synthase I was shown to have a molecular weight of 80,000 ± 5,000 and to be composed of two similarly sized subunits. Synthase II had a molecular weight of 85,000 ± 5,000 and also was apparently homodimeric. Gel electrophoresis of partial proteolytic digests demonstrated that synthases I and II share few if any common peptides. Synthases I and II also were shown to be unrelated by immunological criteria. An improved assay for β-ketoacyl-acyl carrier protein synthase activity gave kinetic parameters for synthases I and II at both 27°C and 37°C using five long chain acyl-acyl carrier protein substrates. The properties of synthase II are consistent with the proposed role of this enzyme in the modulation of fatty acid synthesis by temperature. *fabF* mutants of *E. coli* lack synthase II. The *fabF* locus was mapped at min 24.5 of the *E. coli* genetic map and the clockwise map order was found to be *pyrC, fabD, fabF, purB*.

Unsaturated fatty acids comprise about one-half the fatty acid content of *Escherichia coli* and are primarily found esterified to position 2 of the *sn*-glycerol 3-phosphate backbone of the membrane phospholipids (for review, see Ref. 1). Palmitoleic (C16Δ^{9}) and *cis*-vaccenic (C18Δ^{11}) acids are the sole unsaturated fatty acids found in this organism, whereas palmitic acid (C16:0) is the major saturated fatty acid. The fatty acid composition of *E. coli* changes as a function of growth temperature (2), the proportion of unsaturated fatty acids increasing with lower growth temperature. In *E. coli* this adaptive response does not involve *de novo* enzyme synthesis (3), and the increased amount of unsaturated fatty acid produced at lower growth temperature corresponds to an increased rate of synthesis of *cis*-vaccenic acid (4–6). The primary site of temperature regulation is at the level of fatty acid synthesis (7).

In *E. coli*, the chain elongation step of fatty acid synthesis is the condensation of an acyl group bound to acyl carrier protein (ACP)[1] with malonyl-ACP (8). This reaction is catalyzed by the enzyme, β-ketoacyl-ACP synthase, which can be separated into two forms, synthase I and synthase II (9). The two forms differ in their pH optima, heat lability, and molecular weight (9). We have recently shown that *fabF* mutants of *E. coli*, which are deficient both in the temperature regulation of fatty acid synthesis and in the elongation of palmitoleic acid to *cis*-vaccenic acid (10), lack β-ketoacyl synthase II (11). D'Agnolo and co-workers (9) had previously reported that a class of mutants (*fabB*), deficient in overall unsaturated fatty acid synthesis, lack β-ketoacyl-ACP synthase I. We further demonstrated that the *fabB* locus is the structural gene for β-ketoacyl-ACP synthase I (11). Since *fabF* mutants possess synthase I activity, *fabB* mutants possess synthase II activity, and *fabF fabB* double mutants lack all fatty acid elongation activity (11), it was considered likely that synthases I and II are distinct enzymes and the products of different structural genes (11). However, the two enzyme forms co-purify through several protein fractionation steps (2) and have similar properties. Thus, it seemed possible that synthase II is a modified form of synthase I. This putative modification could be involved in the temperature control of fatty acid composition of the membrane phospholipids of *E. coli*, since synthase II has a key role in temperature control (11). Therefore, we have purified the two synthases and compared their properties.

In this paper, we report conclusive evidence that β-ketoacyl synthases I and II have different primary structures. An improved assay for β-ketoacyl-ACP synthase activity is reported, and the substrate specificities of synthases I and II were analyzed at two different temperatures with five long chain acyl-ACP substrates. The relevance of these data to the regulation of fatty acid synthesis is discussed. The *E. coli* genetic map location of the *fabF* gene, the presumptive structural gene for β-ketoacyl-ACP synthase II, has also been determined.

EXPERIMENTAL PROCEDURES[2]

Materials [^{14}C]Decane-1,3-diol and [^{14}C]-tetradecane-1,3-diol (both labeled in the odd numbered carbon atoms) were synthesized from 8-[^{14}C]hydroxydecanoyl-N-acetylcysteamine and 8-[^{14}C]hydroxymyristic acid, respectively. The 8-[^{14}C]hydroxydecanoyl-N-acetylcysteamine was reduced to the diol with NaBH$_4$ in 30% (v/v) tetrahydrofuran (see below). 8-[^{14}C]Hydroxymyristic acid was reduced to the diol with a 5-fold excess of LiAlH$_4$ in tetrahydrofuran.

8-[^{14}C]Hydroxydecanoyl-N-acetylcysteamine was synthesized from the labeled acid and N-acetylcysteamine as described by Kass and Breck (12). 8-[^{14}C]Hydroxydecanoic acid was isolated from the rhamnolipid of *Pseudomonas aeruginosa* grown in the presence [1-^{14}C]acetate (13). In a similar manner, 8-[^{14}C]hydroxymyristic acid was isolated from an *E. coli* mutant (14) grown in the presence [1-^{14}C]acetate. In both cases the acid was liberated by acid hydrolysis and purified as the free acid by thin layer chromatography.

All nonradioactive fatty acids were purchased from Nu Chek Prep, Elysian, MN, except cis-3-decenoic, cis-5-dodecenoic, and cis-7-tetradecenoic acids which we synthesized as follows. 3-Decynoic acid was synthesized from 3-decyn-1-ol (Chemical Samples Co., Columbus, OH) by oxidation with chromic acid (15). After oxidation, the neutral fraction was saponified to liberate the acid moiety of the ester formed as side product of the oxidation (16). After crystallization twice from pentane, 3-decynoic acid (mp 38.4–39°) was obtained in 30% yield.

* This research was supported by National Institutes of Health Grants AI 10186 and AI 15650. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ACP, acyl carrier protein; *tet*^{R}, tetracycline-resistant; SDS, sodium dodecyl sulfate.

[2] Portions of this paper (including "Experimental Procedures," Figs. 1 to 4, and Tables 1 to 3) are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80M-1516, cite author(s), and include a check or money order for $1.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

5-Dodecynoic acid was synthesized by malonic ester elongation (17) of the mesylate of 3-decyn-1-ol. The mesylate was synthesized as described by Crossland and Servis (18). The acid was an oil at room temperature.

7-Tetradecynoic acid was synthesized from 1-octyne (Chemical Samples Co.) and 6-bromohexanoic acid (Aldrich Chemical Co.) by condensation in hexamethylphosphortriamide-butyl lithium (19). The acid was crystallized from isooctane after addition of seed crystals provided by M. R. Gilman (Hoffman La Roche, Inc., Nutley, N.J.) and melted at 28-29° C.

The ynoic acids were reduced to *cis* isomers using the catalyst of Cram and Allinger (20) and H_2 generated by cleavage of $NaBH_4$ using the apparatus described previously (21). In all cases H_2 uptake ceased after reduction to the monoene was complete. The resulting acids were methylated (10) and purified free of any starting material, saturated acid, or *trans* isomer by argentation thin layer chromatography (10). The purity of the esters was also assayed by gas chromatography (10) and was found to be >98% in all cases. After purification, the esters were saponified to give the potassium salt of the acid.

Malonyl transacylase was prepared as described by Greenspan *et al*. (22) and assayed radiochemically as described by Ruch and Vagelos (23). The malonyl transacylase preparation was treated with 100 µM iodoacetamide in 0.1 M potassium phosphate for 5 min at 22° to inactivate residual β-ketoacyl ACP synthase activity (24). A unit of transacylase activity is 1 µmol of malonyl-ACP formed/min.

Acyl carrier protein (ACP) was prepared according to the method of Majerus *et al*. (25) with the following modifications. The supernatant resulting from resuspension of the acid precipitate was mixed with an equal volume of 2-propanol, centrifuged (5,000 x g for 10 min) and the supernatant loaded directly onto a DEAE-cellulose column (26). The ACP eluted from the DEAE column (detected by the acyl-ACP synthetase assay) was found to be homogeneous by sodium dodecyl sulfate electrophoresis in the presence of 8 M urea. Contaminating nucleic acid was removed by repeated isoelectric precipitation (26, 27). ACP concentrations were determined as described previously (28). Acyl-ACP derivatives were synthesized with acyl-ACP synthetase (28-31) and purified by hydrophobic chromatography (28, 31).

Staphylococcus aureus V8 protease was obtained from Miles Laboratories, Elkhardt, IN, papain and chymotrypsin (tosylleucylchloroketone treated) were obtained from EM Laboratories, Elmsford, NY. The electrophoresis reagents were from Bio-Rad Laboratories, Richmond, CA.

Purification of β-Ketoacyl-ACP Synthases I and II

Cells of strain UC1 (238 gm) grown to late log phase on a rich broth medium were used. The purification procedure was that of Prescott and Vagelos (32) with the modifications starting at the hydroxylapatite step introduced by D'Agnolo *et al*. (9). A column of hydroxylapatite (BioRad HTP without inclusion of Sephadex G-25) was use to separate synthases I and II. No separation was obtained using Hypatite-C (Clark Chemical Co., Williamsport, TN), a porous preparation of hydroxylapatite. Each of the separated synthases was then purified to homogeneity by gel filtration on Bio-Gel P200.

β-Ketoacyl-ACP Synthase Assay

The synthase assay (total volume 20 µl) contained 0.2 M potassium phosphate (pH 6.8); sodium ethylenediaminetetracetic acid, 1 mM; dithiothreitol, 0.3 mM; malonyl transacylase, 30 mU/ml; ACP, 40 µM; [2-^{14}C] malonyl-CoA (10 Ci/mol), 100 µM; acyl-ACP (usually tetradecanoyl), 60 µM and β-ketoacyl-ACP synthase. For maximal activity the malonyl transacylase and ACP were reduced before the assay by incubation of a mixture of these two components plus the ethylenediaminetetracetic acid and dithiothreitol in 0.1 M K_2HPO_4 for 10-15 min at 37° C. An equal volume of 0.1 M KH_2PO_4 and the malonyl-CoA were then added and the mixture was incubated at room temperature for a few min to allow malonyl transacylase to reach equilibirum. The reaction was then started by addition of acyl-ACP and enzyme. After incubation at 37° C for 20 min (or 27° C for 40 min), the reaction was terminated by addition of 0.4 ml of reducing reagent. The reducing reagent was modeled on the results of Barron and Mooney (33) and contained 0.1 M K_2HPO_4, 0.4 M KCl, 30% tetrahydrofuran, and 5 mg/ml $NaBH_4$, the $NaBH_4$ was added just before use. The reducing solution was stored on ice and used within 2 hr after preparation.

Following addition of the reducing reagent, the contents of the assay tubes were mixed vigorously and incubated at 37° C for at least 30 min. Toluene (0.4 ml) was added and the contents of the tubes were again mixed. The upper phase of the resulting two phase mixture had a volume of 0.51 ml (due to partition of tetrahydrofuran into the toluene). A portion (up to 0.3 ml) of the upper phase was taken and counted directly in a toluene based scintillation solution. A unit of synthase activity catalyzes the incorporation of 1 mol of [2-^{14}C] malonate into the reduced product per min at 37° C. The assay was linear until about 0.35 µmol of product is formed. This corresponds to the conversion of approximately 20 and 30% of the malonyl-CoA and acyl-ACP, respectively.

Immunological Procedures

A New Zealand white rabbit was injected with an emulsion of 1 mg of homogeneous synthase I in Freund's complete adjuvant, (Difco Laboratories, Detroit, MI). Injection was into each hind foot pad and between the scapula. A secondary immunization was given 38 days after the primary injection. After 11 more days the serum was collected and the IgG fraction purified by standard techniques (34) except that a QAE-Sephadex column run in 40 mM Tris-Cl (pH 8.0) was used rather than the usual DEAE cellulose column (the suggestion of E. M. Voss, University of Illinois).

Micro Ouchterlony plates were run on microscope slides as previously described (36). After washing to remove soluble proteins, the protein precitates were stained with Comassie Blue (36).

Thin Layer Chromatography

Silica gel G thin layer plates (500 um thick) were purchased from Analtech, Newark, DE. For separation of long chain diols from mono-ols the solvent systems were petroleum ether:ether:acetic acid (80/20/1, by volume) or ether:28% ammonium hydroxide (100/0.5, by volume).

For separation of fatty acid methyl esters, silica gel plates were dipped into a solution of 20% (w/v) $AgNO_3$ in acetonitrile. The impregnation of the silica gel with $AgNO_3$ was found to be equivalent to 15% impregnation using the usual procedure. After evaporation of the solvent the plates were activated at 100° for at least 2 hr. Plates prepared in this manner and developed once in toluene at -17° C gave highly reproducible separations of palmitoleic, *cis*-vaccenic and saturated acids equivalent to those reported previously (10).

Sedimentation Equilibrium

The method used was as described previously (27). Both synthases and the four largest standards were quantitated by enzymatic analysis, the remaining standards by A_{280}. The buffers used were 0.1 M potassium phosphate, pH 6.8 in the synthase I experiments and 20 mM N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid buffer containing 0.1 M ammonium sulfate (adjusted to pH 7.1 with KOH). Both buffers contained 10 mM 2-mercaptoethanol and 10 mg/ml Dextran T-10. The synthase II samples also contained ovalbumin (0.2 mg/ml) to prevent the adsorption of the enzyme to the centrifuge tube (6). The synthase preparations were hydroxylapatite purified preparations from strains UC1 (synthase I) or K1060 (synthase II). Similar values (± 5,000) were obtained in four different experiments with synthase I and three different experiments with synthase II. The appropriate control experiments showed no loss of synthase activity during centrifugation. The $\bar{v}$ for synthase I and II (both = 0.73) were calculated from the amino acid compositions (Table 3).

Genetic Methods

The strains used are given in Table 1. All *fabB*^{ts} strains were constructed by mating a streptomycin resistant F⁻ strain with the Hfr strain CY222. Strain CY222 is an HfrC *fabB*^{ts}, *gyrA* strain derived from strain L35-2 of Baldassare *et al*. (36) by transduction of the *fabF* allele to *fabB*15, a temperature sensitive allele. After a 3 hr incubation, the mating mixtures were plated on media containing naladixic acid (100 µg/ml) and streptomycin (200 µg/ml) at 33.5°. About half of the *gyrA* recombinants inherited the *fabB*15 allele.

Transductions were performed as described previously (37) except that phage adsorption was at 33° for 30 min. In addition to the usual controls, in transductions using the *fabD* marker of strain LA2-89 as a recipient, a parallel cross using phage grown on strain LA2-89 was also performed. This cross was used to determine the killing action of phage infection on the spontaneous revertants that frequently occurred in cultures of strain LA2-89.

For experiments using the Tn10 insertion element, the media contained tetracycline HCl at 10-20 µg/ml. The methods used to select for transposition were given previously (38, 39).

Scoring of Transductants

In a *fabB*^{ts} background, the *fabF* locus is readily scored (11). However, since *fabB* mutants have the same growth phenotype as *fabB fabF* double mutants, the linkage of *fabF* with *fabB* was scored by analysis of the fatty acid composition.

The transductants were purified by replating at 42° at least twice and were then grown in a defined medium (10) overnight at 30°. These cultures were diluted 10-fold and grown for 1 hr at 30° before addition of 2 µCi/ml of Na [1-^{14}C]acetate (46-60 Ci/mol). After an additional 45 min incubation, the phospholipids were extracted from the cultures and the fatty acid moieties converted to their methyl esters (40). After argentation thin layer chromatography, the chromatograms were autoradiographed. The Cvc⁻ phenotype was scored by visual inspection of the autoradiograms (6, 10).

Characterization of the Radiochemical Assay for β-Ketoacyl-ACP Synthase

The assay previously used in virtually all the work with the β-ketoacyl-ACP synthases of *E. coli* was a coupled assay(9, 22, 24, 32, 41). The β-ketoacyl-ACP was reduced to β-hydroxyacyl-ACP using either β-hydroxyacyl-CoA dehydrogenase from pig heart or a partially purified β-ketoacyl-ACP reductase from *E. coli*. The coupled assay was then followed by reduction of pyridine nucleotide. Although this assay was sufficient for enzyme purification, the interpretation of substrate specificity studies was quite difficult since the specificities of the coupling enzymes for the various β-ketoacyl-ACP species were unknown.

A second problem with the previous assay is the nature of the ACP substrate. The acyl-ACP substrates were synthesized chemically and this procedure gives rise to substrates of widely varying activity in which the ACP moiety is not in its native conformation (28, 29). Furthermore, the acyl-ACP preparations were contaminated with denatured ACPSH. The problems encountered with these substrates are best illustrated by the wide variation in Michaelis constants reported by one laboratory (9, 22, 24, 32, 41) for various substrates. The Km of synthase I acetyl-ACP has varied from 19-52 µM, that of *cis* 3-decenoyl-ACP from 12 to 71 µM and that of palmitoleoyl-ACP from 37 to 130 µM. We therefore wished to avoid use of chemically prepared substrates.

The radiochemical assay we developed uses native acyl-ACP substrates synthesized by acyl-ACP synthetase (30, 31) and purified free of ACP by hydrophobic chromatography (28, 29). The condensation of the acyl-ACP substrate with malonyl-ACP to give β-ketoacyl-ACP was followed by use of [2-^{14}C]malonyl-ACP. We used $NaBH_4$ treatment to convert the β-ketoacyl-ACP product into a form that could be extracted into organic solvents. $NaBH_4$ treatment converted into β-ketoacyl-ACP into a 1,3-diol by reducing both and carbonyl groups. Barron and Mooney (33) showed that acyl thioesters were readily reduced to a fatty alcohol by treatment with $NaBH_4$ in 30% aqueous tetrahydrofuran. Reduction of the ketone group was desirable due to the instability of β-keto acids.

The cleavage of the thioester was quantitative. After 20 min of reaction of [^{14}C]palmityl-ACP with $NaBH_4$, >90% of the palmitate radioactivity could be extracted into toluene. In the absence of $NaBH_4$ <5% of the radioactivity was toluene extractable. After $NaBH_4$ treatment, the product of the condensation of tetradecanoyl-ACP with [^{14}C]malonyl-ACP cochromatographed with authentic 1,3-diols (Fig. 1). No free fatty acids were formed indicating that no hydrolysis of acyl-ACP occurred under these conditions. Quantitative extraction into toluene was obtained with diols ≥C10.

The assay was linear with time for at least 25 min (Fig. 2) and with purified synthase to at least 0.2 µg of protein (Fig. 3). The assay had obligate requirements for acyl-ACP and malonyl transacylase and was stimulated by dithiothreitol and ethylenediaminetetraacetic acid (Table 2). The activity in the absence of added acyl-ACP can be attributed to the decarboxylation of malonyl-ACP by β-ketoacyl-ACP synthase to form acetyl-ACP (42). The activity in the absence of added ACP can be attributed to traces of ACP in the various protein solutions. As expected from the malonyl-CoA CO_2 exchange reaction (25), ACP acts catalytically in this assay.

The sensitivity of the assay was limited only by the specific activity of the [2-^{14}C]malonyl-CoA available. Malonyl-ACP was generated from malonyl-CoA and ACP in the reaction mixture. The equilibrium of malonyl transacylase is 0.1 thus precluding efficient preparative synthesis of malonyl-ACP from malonyl-CoA (43).

Table 1. Bacterial Strains.

Cell strains are derivatives of *E. coli* K-12. CGSG denotes the Coli Genetic Stock Center, Yale University (New Haven, CT). Strains CY222, L35-2, and MA1008 are HfrC strains. The remaining strains are F⁻ except the F⁺ strains MR1 and UC1. Strains CY235 and CY239 were constructed by conjugation whereas the remaining strains were constructed by transduction with phage P_1 *vir*.

Strain	Relevant Genotype	Source or Reference
UC1	wild type	(10, 11)
MR1	*fabF*	(11)
LA2-89	*fabD*	(45)
AB2829 *pyrC*	wild type	(46)
PC0254	*purB*	CGSG
MA1008	*pyrC*	CGSG
X7014a	*pyrC*, *purB*	CGSG
L35-2	*fabF*, *gyrA*	(36)
CY222	*fabB*^{ts}, *gyrA*	see text
CY232	*fabB*^{ts}, *fabF*, *gyrA*	(11)
CY235	*fabB*^{ts}, *purB*, *gyrA*	CY222 x PC0254
CY239	*fabB*^{ts}, *pyrC*, *purB*, *gyrA*	CY222 x X7014a
CY244	*fabB*^{ts}, *fabF*, *purB*	P_1(MR1) x CY239
CY288	*fabB*^{ts}, *fabF*, Tn10	see text
CY290	*fabB*^{ts}, Tn10	see text
CY291	*fabB*^{ts}, *fabD*	P_1(LA2-89) x CY235
CY292	*fabB*^{ts}, *fabD*, Tn10	P_1(CY290) x CY291
K1060	*fabB*	(47)

Table 2. Requirements of the Radiochemical β-Ketoacyl-ACP Synthase Assay.

The concentrations of reagents of the complete assay mix were: 200 mM potassium phosphate (pH 6.8), 1 mM ethylenediaminetetraacetic acid, 0.3 mM dithiothreitol, 30 mU/ml malonyl transacylase, 40 µM ACP, 100 µM [2-^{14}C] malonyl-CoA (sp. act. 10 Ci/mol), and 50 µM tetradecanoyl-ACP. Each assay also contained 7 microunits of partially purified synthase I. The background activity substracted was the value obtained without incubation.

Reaction mix	Activity
Complete	(100)
Minus dithiothreitol	56
Minus ethylenediaminetetraacetic acid	61
Minus ACP	40
Minus tetradecanoyl-ACP	5
Minus malonyl transacylase	<1
Minus tetradecanoyl-ACP and malonyl transacylase	<1

Table 3. Amino Acid Compositions of β-Ketoacyl-ACP Synthases I and II.

The analyses were performed after a 24 hr hydrolysis of homogeneous synthases I and II (6 μg each) in 6N HCl. The samples were from the E. coli K-12 strain UC1. The compositions are given in mole percent.

Amino Acid	Synthase I	Synthase II
	mole percent	
Asp	8.7	8.0
Thr	5.9	6.0
Ser	8.0	11.8
Glu	10.2	14.2
Pro	3.2	3.3
Gly	12.6	12.2
Ala	12.2	7.6
Val	7.1	6.0
Met	4.2	1.4
Ile	5.0	3.9
Leu	7.2	8.3
Tyr	2.4	3.0
Phe	2.2	2.6
His	2.6	1.9
Lys	4.3	3.8
Arg	4.3	6.1

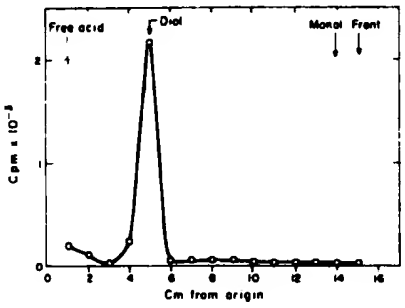

Figure 1. Chromatographic Identification of the Reaction Product.

A sample of the toluene extract of a standard assay was applied to a Silica Gel (Analtech) thin layer plate (250 μ thick). The plate was developed once in a mixture of diethyl ether: concentrated NH4OH (100/1, v/v). The standard were tetradecanoic acid, 1-tetradecanol, and 1,3-tetradecanediol. The substrate for the assay was tetradecanoyl-ACP. The migration of long chain mono- and dihydroxy hydrocarbons in this system was determined to be insensitive to chain length. The standards were visualized by iodine vapor and the silica gel was scraped (in 1 cm wide areas) into scintillation vials and counted in Aquasol. The distances plotted are the furthest point of the 1 cm area from the origin. A similar result was found using the solvent system of petroleum ether:ether:acetic acid (80/20/1 v/v).

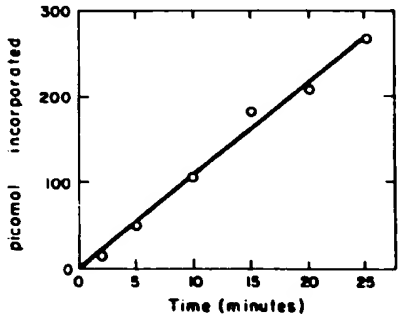

Figure 2. Time Course the β-Ketoacyl-ACP Synthase Reaction.

The reaction mixtures was the same as that in Table 2 (scaled up 10- fold) and contained 1.2 μg of the synthase preparation. At the indicated times, one-tenth of the reaction mix was treated with NaBH4.

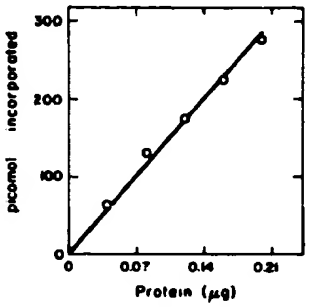

Figure 3. β-Ketoacyl-ACP Synthase Activity as a Function of Protein Concentration.

Standard assay mixtures as described in Materials and Methods were incubated in the presence of different amounts of protein, obtained from an off-peak fraction of wild-type β-ketoacyl synthase from the DEAE cellulose stage of purification (containing both synthases I and II). Tetradecanoyl-ACP was the substrate. The ordinate is the picomol of ^{14}C acetate (from malonyl-ACP) incorporated into β-ketohexadecanoyl-ACP in a 20 min incubation.

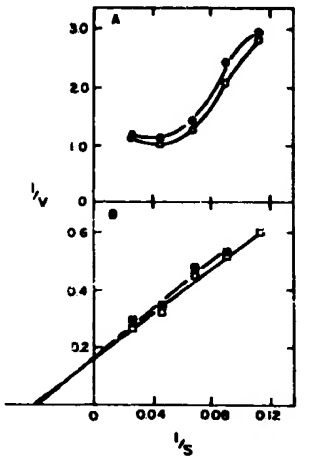

Figure 4. Double Reciprocal Plots of the Reaction of Dodecanoyl-ACP With Synthases I and II

The conditions were as described in Table 4. Panels A and B give the results with two different preparations each of synthase II and synthase I, respectively. The units of 1/v and 1/s are nM and μM, respectively. Lineweaver-Burke (44) plots similar to that given in B were used to obtain the data in Table 4.

RESULTS

Molecular Characterization of Synthase I—We purifed β-ketoacyl-ACP synthase I by a minor modification of the scheme reported by D'Agnolo *et al.* (9) and obtained a preparation having a specific activity of 5.5 units/mg of protein. All synthase I preparations gave a single stained protein band upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (Fig. 5). The apparent molecular weight for synthase I was 43,000 to 44,000. A similar value was found by Phillips and Neidhardt.[3] Neidhardt and coworkers (48–50) have studied the regulation of the synthesis of a large number of *E. coli* proteins using the two-dimensional gel electrophoresis method of O'Farrell (51). By comparison with a sample of synthase I that we provided, Phillips and Neidhart[3] have identified synthase I as their protein F42.2 (48, 49). Protein F42.2 has an apparent molecular weight (in the presence of SDS) of 42,200 and an isoelectric point of about pH 6.0 (estimated from the position of elongation factor Tu[52] on the same gels.[3] Synthase I comprises 0.6% of the protein of *E. coli*. This value does not vary with the growth temperature (49) and increases linearly with increased growth rate[3] (48).

The values that we and Phillips and Neidhart[3] obtained for

[3] T. A. Phillips and F. C. Neidhart, personal communication.

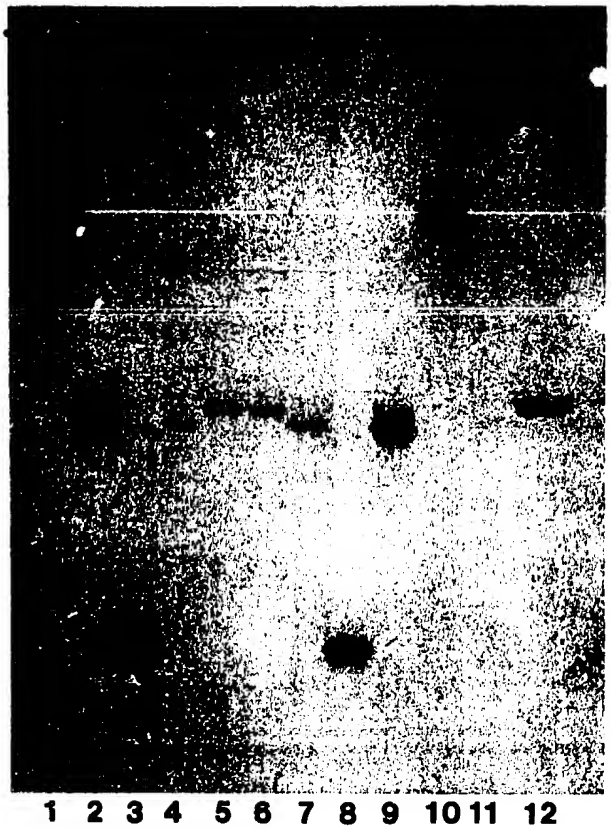

FIG. 5. **SDS-polyacrylamide gel electrophoresis of β-ketoacyl-ACP synthases I and II.** The synthase I and II samples were purified through the gel filtration step of the purification scheme of D'Agnolo *et al.* (9). The samples applied were *Lanes 1* and *10*, bovine serum albumin (M_r = 68,000); *Lanes 2* and *9*, ovalbumin (M_r = 43,000), *Lanes 3* and *8*, bovine carbonic anhydrase (M_r = 30,000); *Lanes 4* and *7*, synthase I from the *E. coli* K-12 strain, UCl; *Lanes 5* and *6*, synthase II from the *E. coli* K-12 strain, UCl; and *Lanes 11* and *12* contained synthases I and II, respectively, from *E. coli* B. The gel system contained 0.1% SDS and was essentially that of Cleveland *et al.* (54). The samples were boiled in the sample buffer of Cleveland *et al.* (54) before loading. The gel was 25 cm in length (0.16 cm thick) and contained 10% acrylamide cross-linked with 0.27% bisacrylamide. Staining and destaining were done as previously described (54).

the molecular weight of synthase I under denaturing conditions are somewhat greater than the values previously reported by Prescott and Vagelos (32). Those workers had obtained molecular weights of 35,000 and 37,000 by SDS-gel electrophoresis and by gel filtration in the presence of guanidine HCl, respectively (32). All of these values are incompatible with the native molecular weight of 66,000 reported by Greenspan and Vagelos (22), and thus, we determined the molecular weight of synthase I by the sedimentation equilibrium method of Bothwell *et al.* (53). The distribution of synthase I as determined by enzymatic activity gave a molecular weight for the active enzyme of 80,000 ± 5,000 (Fig. 6). This value is in good agreement with the average (39,000) of the various determinations of the subunit molecular weight. The amino acid composition we obtained for synthase I (Table 3) agrees well with that previously reported by Greenspan and Vagelos (22) for the enzyme from *E. coli* B.

β-Ketoacyl-ACP synthase II was also purified to homogeneity. Our best preparation had a specific activity of 6.3 units/mg protein and gave a single protein band on SDS-gels (Fig. 5). The apparent molecular weight of synthase II was 44,000 to 45,000, a value slightly (although significantly) larger than that of synthase I. We also determined the molecular weight of the native molecule by sedimentation equilibrium and obtained a value of 85,000 ± 5,000 (Fig. 6). This value indicates that synthase II like synthase I is composed of two subunits of similar or identical molecular weights. By gel filtration an apparent molecular weight for synthase II of 76,500 was obtained by D'Agnolo *et al.* (9). The similarity of this value to that obtained by sedimentation equilibrium argues that synthase II is a globular protein. The amino acid composition of synthase II was similar but not identical to that of synthase I (Table 3).

Comparison of the Primary Structures of Synthases I and II—We tested the relationship between synthases I and II by peptide mapping using the method of Cleveland *et al.* (54). Homogeneous samples of synthases I and II were digested with a protease in the presence of SDS. The digestions were run in parallel and the resulting peptides were separated by polyacrylamide gel electrophoresis in the presence of SDS and urea (Fig. 7).

The peptide maps of synthases I and II obtained with *Staphylococcus* V8 protease, chymotrypsin, and papain were strikingly different. Furthermore, synthases I and II differed greatly in their sensitivity to both *Staphylococcus* V8 protease and papain (Fig. 6). Peptide maps of synthases I and II cleaved with CNBr also differed markedly, but the Coomassie blue staining was too faint for adequate photographic reproduction (data not shown). We conclude that synthases I and II share few if any amino acid sequences.

We have also tested the immunological relationship between synthases I and II (Fig. 8). A purified IgG fraction was

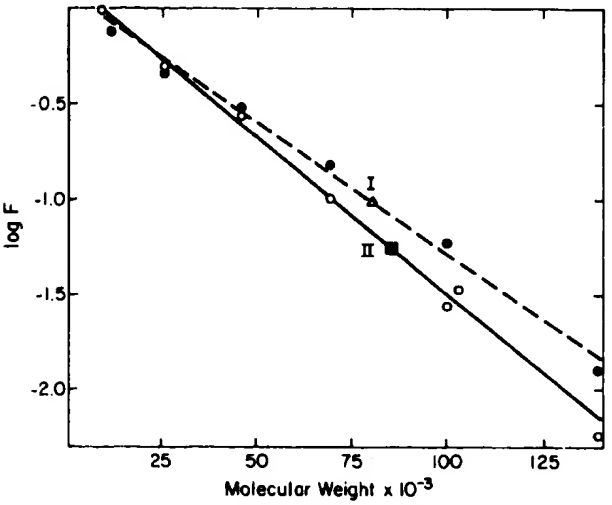

FIG. 6. **Sedimentation equilibrium analysis of synthases I and II.** The experiments were performed essentially as described by Bothwell *et al.* (53) with the modifications previously used in this laboratory (27). The values plotted are the log of the fraction of protein remaining in the top 40% of the tube after centrifugation *versus* the molecular weight of the protein normalized to a partial specific volume of 0.725 ml/g. The upper curve (●– – –●) was an experiment performed on synthase I. The nominal speed of the air turbine centrifuge was 43,000 rpm (for 10 h). In the *lower curve* (○——○), the experiment on synthase II was run at a nominal speed of 50,000 rpm for 8 h. The positions of synthases I and II are given by the symbols Δ and ■, respectively. The standards for synthase I were ACP (M_r = 8,850, $\bar{v}$ = 0.731), chymotrypsinogen (M_r = 25,700, $\bar{v}$ = 0.734), ovalbumin (M_r = 44,600, $\bar{v}$ = 0.744), bovine serum albumin (M_r = 68,000, $\bar{v}$ = 0.735), *E. coli* alkaline phosphatase (M_r = 102,000, $\bar{v}$ = 0.730), beef heart lactic dehydrogenase (M_r = 136,000, $\bar{v}$ = 0.740). The same standards were used for synthase II except horse heart cytochrome *C* (M_r = 11,700, $\bar{v}$ = 0.728) was used in place of ACP and another standard, *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (M_r = 103,600, $\bar{v}$ = 0.718), was included. Further details are given under "Experimental Procedures."

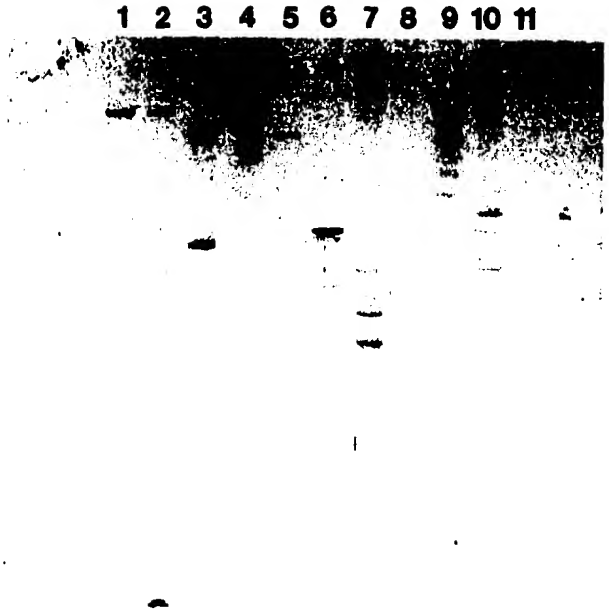

FIG. 7. **Comparative peptide mapping of synthases I and II.** The protease digestions were performed in the presence of SDS essentially as described by Cleveland *et al.* (54). *Staphylococcus aureus* V8 protease and chymotrypsin were used at 50 μg/ml, whereas papain was used at 7.5 μg/ml. The synthase concentrations used were approximately 1 mg/ml and 500 μg/ml for synthases I and II, respectively (the enzymes from the *E. coli* K-12 strain, UCl were used). The protease digestions were incubated for 45 min at 37°C before electrophoresis. The gels used differed from those in Fig. 1 in the thickness (0.08 cm), the length (14 cm), the acrylamide concentration (15%), and that the separating gel contained 8 M urea. *Lane 1*, synthase I undigested; *Lane 2*, synthase II undigested, *Lane 3*, synthase I digested with V8 protease out-*Lane 4*, synthase II digested with V8 protease; *Lane 5*, V8 protease; *Lane 6*, synthase I digested with chymotrypsin; *Lane 7*, synthase II digested with chymotrypsin; *Lane 8*, chymotrypsin; *Lane 9*, synthase I digested with papain; *Lane 10*, synthase II digested with papain out-*Lane 11*, papain. As observed by Cleveland *et al.* (54), the patterns of peptides given with V8 protease and chymotrypsin were quite insensitive to small (≤4-fold) variations in protease concentration. However, the patterns obtained with papain were considerably altered by a 3-fold change in papain concentration.

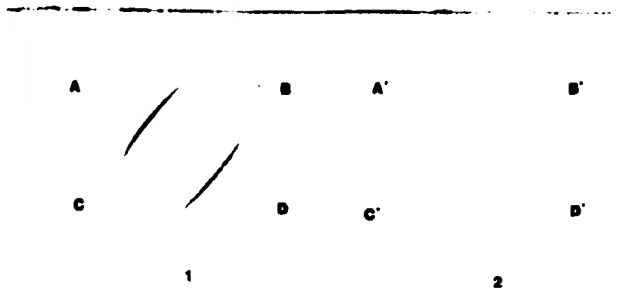

FIG. 8. **Immunological relationship between synthases I and II.** The *center wells* contained 9 μg of antisynthase I IgG (*Pattern 1* at left) or 7 μg of control (preimmune) IgG (*Pattern 2* at right). The outer *Wells A* and *D* of both patterns contained 1.56 munits of synthase I, whereas outer *Wells B* and *C* contained 1.68 munits of synthase II. The enzyme preparations used were purified by the standard procedure except that the final gel filtration step was omitted.

obtained from the serum of a rabbit injected with homogeneous synthase I. The anti-synthase I IgG preparation gave a readily detectable precipitin line with partially purified β-ketoacyl-ACP synthase I, but no precipitin line was detected when equivalent activities (and thus equivalent masses of protein (9)) of synthase II preparations were exposed to the antibody. We conclude that synthases I and II have few antigenic determinants in common.

Substrate Specificities of Synthases I and II—Extensive data on the substrate specificity of β-ketoacyl-ACP synthase I have been reported. However, the kinetic constants for the various substrates differ greatly among the various reports. As discussed in more detail in the miniprint section, we attribute this variability to two defects in the assay method: the specificity of the enzyme catalyzing the coupled reaction that allows the activity to be monitored spectrophotometrically; and the chemical preparation of the acyl-ACP substrates. We have developed a radiochemical assay to avoid the first problem (see "Experimental Procedures" in miniprint section) and use enzymatically synthesized acyl-ACP substrates to avoid the artifacts of chemical synthesis.

The relative activities of synthases I and II can be greatly altered by the assay conditions used (9), and thus we have normalized our maximal velocity data to that obtained with tetradecanoyl-ACP as the substrate (Table IV). We chose tetradecanoyl-ACP because it is an excellent substrate for both synthases *in vitro* and *in vivo* (as argued from genetic evidence (11)).

TABLE IV

Kinetic constants of β-ketoacyl-ACP synthases I and II

The V_{rel} values are apparent V_{max} values expressed relative to the apparent V_{max} of tetradecanoyl (C14:0)-ACP at 37°C. The V_{max} and K_m values were obtained from Lineweaver-Burk (44) plots (Fig. 4). The plots had four or five different concentrations of acyl-ACP evenly distributed in inverse substrate concentration over a 4-fold concentration range. The correlation coefficients (least squares regression) for each data set were >0.98. The V_{max} values with tetradecanoyl-ACP at 37°C were 2.9 and 1.1 units/mg of protein for synthases I and II, respectively. The synthase preparations were purified through the hydroxylapatite step from the *E. coli* K-12 strain, AB2829 *pyrC*. Synthase II did not follow Michaelis-Menten kinetics with C12:0-ACP (Fig. 4).

Acyl-ACP substrate	Synthase I		Synthase II	
	K_m	V_{rel}	K_m	V_{rel}
	μM		μM	
37°C				
18Δ[11]	ND*	<0.005	ND	<0.005
16Δ[9]	267	0.19	216	2.33
14Δ[7]	27	1.97	60	3.00
12Δ[5]	28	1.86	24	1.36
16:0	ND	<0.005	ND	<0.005
14:0	71	(1.0)	68	(1.0)
12:0	22	1.62	ND	ND
27°C				
16Δ[9]	54	0.02	97	0.67
14Δ[7]	40	0.90	43	0.84
12Δ[5]	72	2.00	24	0.74
14:0	128	0.72	47	0.56
12:0	27	1.31	ND	ND

* ND means the value could not be determined.

Both β-ketoacyl-ACP synthases I and II were essentially inactive with *cis*-vaccenoyl-ACP and palmitoyl-ACP (<1% of the activity with C14:0-ACP). This is consistent with the finding that *E. coli* contains only traces (if any) of the final elongation products (*cis*-11 eicosenoic acid and stearic acid, respectively). Both synthases funtioned with all of the other substrates tested although there was one striking difference between the two enzymes in that palmitoleoyl-ACP was an excellent substrate for synthase II but a poor substrate for

synthase I (Table IV). This difference was primarily due to the slow rate at which synthase I elongated this substrate, as the Michaelis constants of the two enzymes for palmitoleoyl-ACP were similar.

We have proposed that β-ketoacyl-ACP synthase II is intimately involved in the temperature regulation of fatty acid composition in *E. coli* (11), and thus we tested the effect of a decreased assay temperature on the kinetic constants of both synthases. As expected, at 27°C with palmitoleoyl-ACP as the substrate, the difference between synthase I and II was greater than that found at 37°C (Table IV). Although both enzymes had lower K_m values for palmitoleoyl-ACP at the lower temperature, the relative velocity of the synthase II reaction was disproportionately greater.

Genetic Analysis of a Synthase II Mutant—Although *fabF* mutants lack β-ketoacyl-ACP synthase II, these strains grow normally (10). However, if a temperature-sensitive *fabB* mutation (*fabB*^{ts}) is introduced into a *fabF* strain, these double mutants are unable to grow on media supplemented with oleate at 42°C (11) (*fabB*^{ts} mutants grow well at 42°C if supplemented with oleate). This growth phenotype was used to locate the *fabF* locus on the genetic map of *E. coli*.

Interrupted matings of a *fabF*, *fabB*^{ts} strain, CY216, with several different *fab*$^+$ Hfr strains were carried out by the method of Zipkas and Riley (55). These experiments indicated that the *fabF* gene was located near min 24 of the current genetic map of *E. coli* (56). Finer mapping was carried out by transduction with phage P1 (Table V). The *fabF* locus was co-transduced almost equally (22 to 27%) with two markers in this region, *pyrC* and *purB* (Table V). The *pyrC* and *purB* loci are only a few per cent co-transduced (45, 56), and thus, the *fabF* gene must be located approximately midway between these two genes.

Another lesion in fatty acid synthesis, the *fabD* gene that codes for malonyl transacylase, has been mapped between the *pyrC* and *purB* loci by Semple and Silbert (45). We mapped the *fabF* locus in relation to the *fabD* locus using phage P1 stocks grown on a *fabF* strain to transduce a *fabD* strain to temperature resistance. The phospholipid fatty acid compositions of the *fabD*$^+$ recombinants were then analyzed by thin layer chromatography. *fabF* mutants are sufficiently deficient in *cis*-vaccenate synthesis that this deficiency is readily scored by visual inspection of autoradiograms of the thin layer chromatograms (6, 10). These experiments demonstrated that the *fabF* and *fabD* genes are tightly (89%) linked (Table V).

The order of the *fabF* and *fabD* genes on the genetic map was determined in relation to the *purB* locus. *fabD* mutants have the same growth phenotype as *fabF*, *fabB*^{ts} strains (45), and since the *fabF* growth phenotype depends on having a *fabB*^{ts} lesion in the same strain (11), elaborate conventional strain construction would have been needed to establish the map order. To simplify the strain construction and analysis, a strain carrying a Tn10 transposon integrated very close to the *purB* locus was used. This strain was isolated by selecting simultaneously for purine-independent and tetracycline-resistant (*tet*^{R}) recombinants of the *purB* strain, PC0540, with P1 phage grown on a pool of random Tn10 insertions (38, 39). The Tn10 insertion used was >89% linked to the *purB* locus (Table V).

Strains were constructed carrying the Tn10 insertion and either *fabD* or *fabF*. P1 phage grown on these strains were used to infect either a *fabF* or a *fabD* strain. All recipient and donor strains carried a *fabB*^{ts} mutation so that the *fabF* genotype could be scored by its growth phenotype. Equal volumes of each transduction mixture were plated on two plates containing tetracycline. One plate was incubated at 30°C to select for tetracycline resistance (*tet*^{R}) and the other was incubated at 42°C to select for *tet*^{R}, *fabD*$^+$*fabF*$^+$. The results of these crosses (Table V) show that if *fadD* was carried by the donor and *fabF* by the recipient, 22% of the tet^R recombinants were *fabD*$^+$*fabF*$^+$, whereas in the reverse cross (*fabF* in the donor), <0.3% of the *tet*^{R} recombinants were *fadD*$^+$*fabF*$^+$. The latter result is that expected for a four cross-over class of recombinants whereas the former result is that expected for a two-cross-over class. These data are only consistent with the order *fabD*, *fabF*, Tn10. Since the Tn10 insertion used was very tightly linked to *purB* (Table V), the clockwise map order must be *pyrC*, *fabD*, *fabF*, *purB*.

DISCUSSION

β-Ketoacyl-ACP synthases I and II of *E. coli* are two distinct proteins. Synthase I is coded by the *fabB* gene (11)

TABLE V

Transductional mapping of the fabF gene

All strains except WN1, LA2-89, PC0254, and MA1008 also carry a *fabB*^{ts} lesion (*fabB21*). The two factor crosses were performed and scored by standard procedures except Cross 3 in which *fabF* was scored by fatty acid analysis (see text). In Crosses 8 and 9, equal volumes of a single transduction mixture were plated on two plates containing tetracycline. The plates were incubated at 30°C for 12 h, then one of the two plates was shifted to 42°C. The 30°C plate gave the number of *tet*^{r} recombinants whereas only *tet*^{r} *fabD*$^+$*F*$^+$ recombinants grew on the 42°C plate. The same procedure was used for Crosses 10 and 11. The medium used in Crosses 8 to 11 was R broth containing Medium E and 10 μg/ml of tetracycline-HCl.

Cross	Bacterial strains and relevant markers		Selected markers	Colonies scored	Co-transduction frequency
	Donor	Recipient			%
Two-factor crosses					
1	WN1 *fabF*	CY235 *purB*	*pur*$^+$	169	22.5
2	WN1 *fabF*	CY239 *pyrC*	*pyr*$^+$	212	26.4
3	CY244 *fabF*	LA2-89 *fabD*	*fabD*$^+$	132	88.8
4	CY290 Tn10	PC0254 *purB*	*tet*^{r}	67	>99
5	CY290 Tn10	MA1008 *pyrC*	*tet*^{r}	183	3.3
6	CY290 Tn10	CY232 *fabF*	*tet*^{r}	38	39.5
7	LA2-80 *fabD*	CY235 *purB*	*pur*$^+$	38	18.4
				Colonies formed	Recombination frequency %
Three factor crosses					
8	CY292 *fabD*, Tn10	CY232 *fabF*	*tet*^{r}	309	(100)
9	CY292 *fabD*, Tn10	CY232 *fabF*	*tet*^{r}, *fabD*$^+$*F*$^+$	67	21.7
10	CY288 *fabF*, Tn10	CY291 *fabD*	*tet*^{r}	359	(100)
11	CY288 *fabF*, Tn10	CY291 *fabD*	*tet*^{r}, *fabD*$^+$*F*$^+$	0	<0.3

and is a dimer of molecular weight 80,000 (Fig. 6) with two similar, probably identical (32) subunits (Fig. 1). Synthase I readily catalyzes all the condensation reactions of long chain fatty acid synthesis except the elongation of palmitoleoyl-ACP (Table I). Previous workers had reported that synthase I has a molecular weight of 66,000 by sedimentation equilibrium (22) whereas the apparent subunit molecular weight was 35,000 to 37,000 (32). Our subunit molecular weight (44,000 to 45,000) was obtained by SDS-polyacrylamide gel electrophoresis on slab gels, a method more reliable than the early version of the technique used previously (32). A larger discrepancy occurs between our value for the native molecular weight, 80,000 (Fig. 6), and the previous value (22) of 66,000. Although both values were obtained by sedimentation equilibrium, we used the method of Bothwell *et al.* (53) and determined the distribution of the protein by enzymatic activity, whereas the previous workers (22) assayed the total protein distribution by ultraviolet scanning. Heterogeneity was evident in the untraviolet scan for protein reported (22). Since ultraviolet scanning is an insensitive assay for heterogeneity (57), considerable heterogeneity may have been present. The conditions used in the sedimentation experiment of Greenspan and Vagelos (22) were later shown (32) to result in structural changes in the protein (probably dissociation into monomers). It should be noted that our molecular weight estimate for synthase I is compatible with previous sedimentation velocity (22) and gel filtration (9) data and together with these data indicate a globular shape for β-ketoacyl-ACP synthase I.

β-Ketoacyl-ACP synthase II has a molecular weight of approximately 85,000 (Fig. 6) and is composed of two similarly sized subunits (Fig. 5). The subunits are probably identical, on the basis of data reported by Prescott and Vagelos (32). Tryptic digestion of synthase I gave a peptide map with 23 strongly staining spots and a similar number of lightly staining spots (32). Because the synthase I was purified by batch elution from hydroxylapatite rather than by gradient elution, contamination with synthase II was likely.[4] The two synthases have similar lysine plus arginine contents (Table 3) and thus it seems probable that the lightly staining peptides were derived from synthase II. The number of these peptides is only consistent with a homo-dimeric native structure. The simplicity of our partial peptide digestions (Fig. 7) is also consistent with homodimeric structures for both β-ketoacyl-ACP synthases. The similarity of the molecular weight values obtained by sedimentation equilibrium (Fig. 6) to that previously inferred from gel filtration (9) indicates the synthase II is a globular protein.

The data presented in this paper further support our hypothesis that β-ketoacyl-ACP synthase II plays a major role in the thermal regulation of fatty acid synthesis. Physiological studies indicated that an increase in the rate of *cis*-vaccenate synthesis is the primary response of fatty acid synthesis to a decrease in temperature (4–6). This change is accomplished by changes in the activity of a pre-existing enzyme(s) (3). *fabF* mutants, which do not elongate palmitoleate and do not thermoregulate their fatty acid composition, lack synthase II (11). Revertants of *fabF* simultaneously exhibit normalization of fatty acid composition, thermoregulation, and β-ketoacyl-ACP synthase II activity (11). We report here that at 37°C, synthase II elongates palmitoleoyl-ACP with a relative velocity 12-fold more rapid than synthase I (Table IV). At 27°C, the differential is more than 30-fold. In addition, the apparent K_m at 27°C is significantly lower than at 37°C. The changes in kinetic parameters for synthase II are not only consistent with a major role in temperature regulation. They are also consistent with our finding that changes of intrinsic enzyme activity, rather than *de novo* synthesis or enzyme modification, are the basis for the temperature regulation of fatty acid synthesis in *E. coli* (3).

β-Ketoacyl-ACP synthase I is essential for unsaturated fatty acid biosynthesis (9) and thus synthase I catalyzes a reaction in unsaturated fatty acid synthesis that synthase II cannot. The identity of this reaction remains unknown. The most probable site for the unique role of synthase I in unsaturated fatty acid synthesis is the elongation of *cis*-3-decenoyl-ACP. We have argued that synthase I should be very active on this substrate, whereas synthase II should be inactive (11). Unfortunately, we have been unable to synthesize significant amounts of *cis*-3-decenoyl-ACP using either acyl-ACP synthetase (28) or the transacylation activity of synthase I (58). It has been reported that synthases I and II both catalyze the elongation of *cis*-3-decenoyl-ACP samples synthesized by chemical means (9). However, these substrates lack native structure (28, 29) and thus a definitive test of our hypothesis must await the synthesis of native *cis*-3-decenoyl-ACP.

We have shown that the *fabF* locus is very closely linked to the *fabD* locus, the structural gene for malonyl transacylase (Table V). The linkage is sufficiently close that *fabF* and *fabD* could be neighboring genes (59) and thus coordinately controlled. If, as seems likely, the *fabF* locus is the structural gene for β-ketoacyl-ACP synthase II, coordinate synthesis of malonyl transacylase and synthase II may regulate the relative rates of two consecutive steps of fatty acid synthesis.

Acknowledgments—We thank M. Chow for helpful discussions and Drs. P. Fletcher and G. Davis for the amino acid analysis.

[4] J. L. Garwin, unpublished data.

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